Distribution of ADPase Activity in the Lactating Rat Mammary Gland and Its Possible Role in an ATP Cycle in the Golgi Apparatus

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A Ca^{2+}/Mg^{2+}-stimulated ADPase has been found to occur in the lactating rat mammary gland. The enzyme is membrane associated and occurs in mitochondrial, microsomal, and Golgi apparatus fractions. The pH activity curves for the Golgi apparatus and microsomal fractions display two distinct maxima, one at pH 6.3 and one at pH 7.4. Studies with inhibitors and activators indicate that the enzyme is similar to ADPases found in other tissues and is distinct from the uridine nucleoside diphosphatase previously reported in the mammary Golgi apparatus. The occurrence of ADPase in the Golgi apparatus indicates a possible role for this enzyme in the milk secretory process, while the microsomal enzyme could be involved in extracellular activities.

The synthesis and secretion of milk involves the formation of a unique colloidal calcium–phosphate transport complex—the casein micelle—in Golgi vesicles (1). This protein-based complex provides the majority of the inorganic nutrients required by the developing neonate. An essential feature in the formation of the casein micelle is the binding of calcium by phosphoserine residues of the caseins (2). In earlier studies it was shown that a specific casein kinase, localized in the mammary Golgi apparatus, is responsible for the post-translational phosphorylation of casein (3). The requirement of ATP for casein phosphorylation and the demonstration of an AMP–ATP transport system in the mammary Golgi (4) raised the question of the fate of the kinase reaction product (ADP) in the mammary Golgi. This is particularly so because nucleotide diphosphatase activity in this organelle has been reported to be specific for UDP and to have little or no reactivity toward ADP (5). This paper deals with a study of the distribution and characterization of ADPase activity of the lactating rat mammary gland and the possible relationships of this enzyme to milk secretion because of its occurrence in the mammary Golgi apparatus.

MATERIALS AND METHODS

Materials. Dithiothreitol (DTT),[2] 1-levamisole, sodium metavanadate, oligomycin (mixture of A, B, and C), adenosine-5′-diphosphate di(monocyclohexyl ammonium) salt from equine muscle, uridine-5′-diphosphate Tris salt from yeast, deoxycholate sodium salt, malachite green hydrochloride, P^1,P^5-di(adenosine-5′)pentaphosphate (Ap_5A) sodium salt, 5′-adenylimidodiphosphate (AppNHp) lithium salt, ouabain, polyvinyl alcohol (cold water soluble), and thiamine pyrophosphate (TPP) chloride were purchased from Sigma Chemical Company. Triton X-100 and bovine serum albumin were obtained from Pierce Chemical Company. Sodium azide and sodium fluoride were from J. T. Baker Chemical Company.

Mammary glands. Sprague–Dawley rats, 9–11 days postpartum, were purchased from West Jersey Biological Supplies, Wenovah, New Jersey. They were fed *ad libitium* on the commercial Purina[3] rat chow (Purina, St. Louis, MO) diet. Water was available at all times. After shipment, they were kept with their pups for 1 day before sacrifice. The animals were killed by exsanguination following chloroform inhalation. Mammary tissue was then carefully removed, chilled on ice, weighed, and used for subcellular fractionation.

Tissue fractionation. The procedures were carried out at 4°C unless specified. The Golgi apparatus was isolated according to the method of Morré (6) with slight modifications. Mammary glands were minced and suspended in 3 vol of Medium A (37.5 mM Tris–maleate buffer, pH 6.5, 1 mM EDTA, 5 mM $MgCl_2$, and 0.1 mM DTT) containing 0.5 M sucrose and 1% dextran. They were homogenized for 1 min (position 6) by using a polytron 10 ST homogenizer. The homogenate was squeezed through one and then two layers of cheesecloth to remove unbroken cells and connective tissues. To concentrate the Golgi apparatus, the filtered homogenate was centrifuged at 4000*g* for 15 min in an HB-4 swinging bucket rotor. The resulting pellets were washed once in homogenization

buffer and resedimented at 4000*g*. The friable top one-third of the pellet was resuspended in 1 to 2 ml of Medium A containing 0.5 M sucrose and 1% dextran, layered over 1.5 vol of Medium A containing 1.25 M sucrose and 1% dextran, and centrifuged at 100,000*g* for 30 min. Finally, the 1.25 M sucrose–homogenate interface (Golgi apparatus fraction) was removed, diluted with Medium A containing 0.5 M sucrose and 1% dextran, resedimented at 4000*g* for 15 min and stored resuspended in the same buffer at −80°C. Other subcellular fractions, including nuclei, mitochondria, microsomes, and cytosol, were isolated from the combined supernatants and pellets remaining after the recovery of the Golgi apparatus fraction as described by Bingham *et al.* (3). The subcellular fractions other than the Golgi apparatus were diluted in Medium A with 0.25 M sucrose and were stored at −80°C.

Protein assay. Protein was determined using the Pierce BCA (bicinchoninic acid) protein assay reagent (Pierce Chemical Company, Rockford, IL) with the room temperature protocol. Bovine serum albumin was the standard.

Dialysis of microsomes and the Golgi. Some of the Golgi apparatus and microsomal fractions were dialyzed to remove endogenous Ca^{2+} and Mg^{2+}. EDTA was added to the thawed membrane fraction to a final concentration of 5 mM. About 0.9 ml of the EDTA-treated microsomal fraction was dialyzed against 300 ml of a solution containing 37.5 mM Tris–maleate, pH 6.5, 0.1 mM DTT, and 0.25 M sucrose for 35 h at 4°C, with three changes of dialysis medium. The Golgi apparatus was similarly dialyzed, but the dialysate contained 0.5 M sucrose and 1% dextran instead of 0.25 M sucrose. The dialyzed fractions were stored at −80°C.

Enzyme assays. This section deals with all enzyme assays except those involved in the determination of the kinetic constants of ADPase. NADPH–cytochrome c reductase was determined by the method of Masters *et al.* (7). Succinic dehydrogenase and lactose synthetase were assayed according to the method of Pennington (8) and Palmiter (9), respectively.

Mg^{2+} ADPase, Mg^{2+} UDPase, and Mg^{2+} TPPase (thiamine pyrophosphatase) were measured by the method of Chan *et al.* (10). Each enzyme assay contained 10.0 mM $MgCl_2$, 1.0 mM of the appropriate substrate, and 30 mM Mops (4-morpholinepropanesulfonic acid) at pH 7.4; 50 mM Pipes (piperazinediethanesulfonic acid) was substituted for the pH 6.3 assay.

For studies of the subcellular distribution, samples were not dialyzed since the small amount of Mg^{2+} and EDTA from these fractions would not significantly change the total Mg^{2+} (10.00 to 10.02 mM). The ADPase and UDPase assays were incubated for 15 min with 45–55 μg of protein (cytosol at 250 μg of protein) and had zero time blanks for all subcellular fractions except the Golgi apparatus. These last enzymes were incubated for 50 min with about 15 μg of protein and had 10-min blanks which were necessary for the linear range of enzyme assay. The TPPase assay of all subcellular fractions was incubated for 40 min with protein concentrations similar to those of ADPase and had zero time blanks. All assays were carried out within the linear range of the reactions with respect to incubation time and enzyme concentration, had a total volume of 1.0 ml, and except where noted had an incubation temperature of 25°C. The blanks corrected for any nonspecific activity.

At the end of the incubation time, a 200-μl aliquot of the reaction mixture was added into 800 μl of the malachite green–molybdate–polyvinyl alcohol mixture (10). The inorganic phosphate released from the hydrolysis of the substrate reacts with the malachite green–molybdate–polyvinyl alcohol mixture to form a stable complex. The color was allowed to develop at room temperature for 25 min and the optical density at 630 nm was measured by a DU-7 spectrophotometer. The spectrophotometer's "calibrate" function was used with 200 μl water and 800 μl of the malachite green–molybdate–polyvinyl alcohol mixture in the sample cuvette. This function then automatically subtracts the zero phosphate blank for all samples.

The buffers for the ADPase pH study were sodium acetate below pH 6.0, Pipes between pH 6.0 and 7.5, and Tris above pH 7.5 with additional 500 μM 1-levamisole added to inhibit any alkaline phosphatase activity. Mops was later substituted for Pipes because it has a p*I* closer to pH 7.4 and like Pipes neither affects enzyme activity nor binds divalent cations. For inhibitor and activator studies, the subcellular fraction was incubated with the effector and buffer for 2 min before Mg^{2+} and the substrate were added to start the assay.

Electron microscopy. Subcellular fractions were chemically fixed with 1% glutaraldehyde in Dulbecco's phosphate-buffered saline, washed with 8% sucrose solution, and postfixed with 2% osmium tetroxide solution buffered with 0.1 M sodium cacodylate solution at pH 7.4. Fixed samples were then dehydrated in a graded ethanol series and embedded in an epoxy resin mixture. Thin sections, cut with diamond knives, were stained with lead citrate and uranyl acetate solutions and examined with a Zeiss 10B electron microscope (Zeiss, OberKochen, Germany).

For negative staining, 10- to 20-μl aliquots of dilute suspensions of subcellular fractions were absorbed to carbon/Formvar-coated specimen grids for 1 min, and the grids were washed with 5–10 drops of 2% uranyl acetate solution and dried in air.

Equilibria of the ion-ADP system. The common logarithm of the association constants of $Mg^{2+} + HL^{2-} = MgHL$ is 1.58 and that of $Ca^{2+} + HL^{2-} = CaHL$ is 1.52, where L is the ADP which is given by Silen and Martell (11). The constants are determined at pH 7.4 at 25°C and are converted to dissociation constants (K_d) for the equations given below.

$$[ADP]_{total} = [ADP]_{free} + [M\text{–}ADP]$$

$$[M]_{total} = [M]_{free} + [M\text{–}ADP]$$

$$K_d = ([M]_{free} \times [ADP]_{free})/[M\text{–}ADP]$$

Concentrations of free and complexed metal ions were calculated as previously described (12) and the ranges used are given in the Appendix.

Kinetic constants of rat mammary ADPases. The assays to determine the kinetic constants were performed like the other ADPase assays except the ion and ADP concentrations were allowed to vary. The kinetic constants were determined on dialyzed and nondialyzed subcellular fractions. In each case the kinetic constants agreed with each other. All of the assays to determine the kinetic constants for free Mg^{2+} or free Ca^{2+} have a zero free metal ion concentration included; this was fixed at half of the maximum concentration of ADP used in an assay but no metal ions. All of the assays to determine the kinetic constants for Mg–ADP or Ca–ADP have a zero ion–ADP complex concentration included, which has 10 mM metal ions with no ADP.

Theory and data analysis. Nonlinear regression analyses of standard Michaelis curves and variance of parameters with concentration were carried out using the Abacus program which is based on a Gauss–Newton iterative method and which was developed by Dr. William Damert of the Eastern Regional Research Center. Choices between fits of models and statistical methods of analysis of the nonlinear fits are as described by Meites (13). For plots of v against $[S]$ which deviated from standard Michaelis patterns, data were fitted with equations originally derived from Wyman's theory of thermodynamic linkage (14). These models were subsequently adapted for use in enzyme kinetics experiments (15). The simplest explanation for two apparent K_m or V_{max} values could be two enzymes reacting with the same substrate or two classes of substrate sites on the same enzyme. In the latter case the reaction would be

$$\underset{(V_0)}{E} + nX \overset{k_1^n}{\rightleftharpoons} \underset{(V_1)}{EX_n} + mX \overset{k_2^m}{\rightleftharpoons} \underset{(V_2)}{EX_nX_m}. \qquad [1]$$

Here V_0, V_1, and V_2 represent velocities due to the various forms of the enzyme; and the observed velocity (V_{obs}) can be mathematically described as

$$V_{obs} = V_0 f_E + V_1 f_{EX_n} + V_2 f_{EX_n} X_m, \qquad [2]$$

where V_0, V_1, and V_2 represent velocities due to the respective form times the fraction of the enzyme (f) in that form and the observed

velocity is the sum contributed by all states (or alternatively both enzymes). Following Wyman's (14) derivations:

$$V_{obs} = \frac{V_0}{1 + k_1^n} + \frac{V_1 k_1^n X^n}{1 + k_1^n X^n} + \frac{V_2 k_2^m X^m}{1 + k_2^m X^m}. \quad [3]$$

The above expression is valid for sequential binding (or reactivity). For values of n or $m > 1$, k_1 and k_2 represent average k values for a class of binding sites and also contain k_{cat} in the same fashion that K_m does. Since reactions are carried out under Michaelis conditions X represents the total substrate concentration (e.g., M^{2+} free or M–ADP). This model also implies no interaction between sites (enzymes) as a result of binding. Models with interactions did not improve the statistical fit of the data (13).

RESULTS AND DISCUSSION

pH and activity. In a study of the general acid phosphatase activity of the rat mammary gland (16) some ADPase activity was found to be associated with membrane-derived fractions. Preliminary experiments at pH 7.4 showed a threefold stimulation of ADPase by Mg^{2+} for the microsomal fraction. In the case of the Golgi apparatus only minor stimulation was observed using a zero and 15-min two-point assay. A series of time and enzyme concentration studies revealed a hysteretic response with a long lag time (5.88 ± 0.81 min) followed by essentially linear velocity (Fig. 1). The nonlinear behavior was not observed at pH 7.4 for the microsomal enzyme which exhibited linear initial velocities (Fig. 1). Such a hysteretic response can be attributed to substrate-induced conformational changes (15, 17). For all Golgi apparatus ADPase assays, only the linear final velocities were used.

In this work, membrane-associated fractions (Golgi apparatus and microsomes) of the lactating mammary rat gland were studied to determine their pH profiles for Mg^{2+}-stimulated hydrolysis of ADP. Bimodal profiles were obtained for both fractions as shown for the Golgi apparatus in Fig. 2. This is in contrast to the symmetrical pH profile centered at 6.5 as reported for UDPase activity in the lactating rat mammary gland Golgi apparatus (5). In fact, previous reports on the nucleotide specificity of membrane-associated nucleoside diphosphatase activity (NDPase) in Golgi apparatus of various tissues (18–20) have indicated a distinct lack of activity toward ADP. Interestingly, most of these studies have dealt with Triton X extracts of membrane preparations. ADPase activity at pH 6.3 and 7.4 appeared rather constant for three different microsomal and Golgi apparatus preparations (Table I), and the lag time observed in the Golgi at pH 7.4 is also present at pH 6.3. Preincubation of the two membrane preparations with 0.1% Triton X-100 dramatically lowered ADPase activity at pH 6.3 in microsomes and the Golgi apparatus while having the opposite effect on UDPase activity. At pH 7.4 ADPase activity was higher than at pH 6.3 and less inhibited by the detergent for both Golgi and microsomal fractions. On the other hand UDPase activity was lower at pH 7.4 in the Golgi and partially inactivated by Triton X-100, while for the

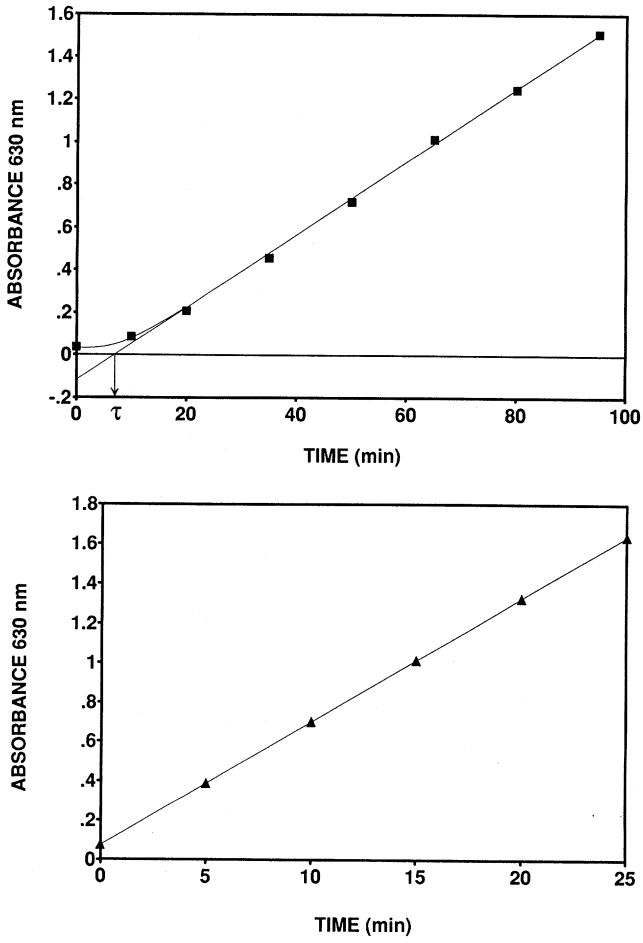

FIG. 1. Time course of the ADPase reaction for enzyme from the lactating rat mammary Golgi apparatus (top). There is an apparent lag time before linear kinetics are achieved. Extrapolation of the linear portion of the curve to the intercept at zero activity yields the lag time (τ) of the reaction (15). The microsomal fraction (bottom) displays linear kinetics under the same conditions.

microsomal fraction UDPase was higher at pH 7.4. Thus it appears that the lack of reported ADPase activity in the rat mammary gland (5) may be due to the use of detergent in the purification schemes coupled with the nonlinearity of the assay for the Golgi apparatus enzyme which would also lead to lower values. Deoxycholate had a similar inhibitory effect on ADPase activity at both pH 6.3 and 7.4, even though it has been used to solubilize ADPase activity from plasma membrane preparations (21).

Subcellular distribution of ADPase activity. Subcellular fractions of the lactating rat mammary gland were isolated by differential centrifugation and assayed for ADPase activity (Table II). Lactose synthetase, NADPH–cytochrome c reductase, and succinate dehydrogenase were used as marker enzymes for the Golgi apparatus, microsomes, and mitochondria, respectively. All membrane-associated fractions showed increased specific activity over homogenate for ADP hydrolysis, with the greatest increase occurring for the microsomal fraction.

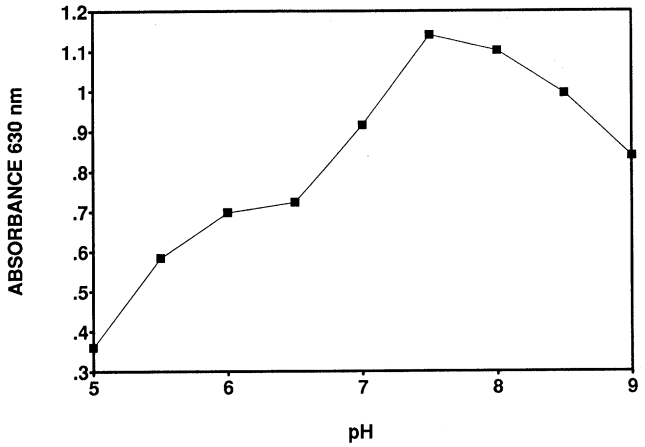

FIG. 2. pH activity curve for Mg^{2+}-stimulated ADPase in the lactating rat mammary Golgi apparatus (■). The standard assay is as described under Materials and Methods.

ADPase has been reported to occur as an ecto enzyme on the plasma membranes of a number of tissues such as heart, lung, and smooth muscle (21–23). In addition this enzyme, found on the outer surface of erythrocytes, is thought to limit ADP-mediated platelet aggregation (24). Thus, it is not surprising that specific activity would be high in the microsomal fraction of the rat mammary gland. This fraction contains membranes from both secretory epithelial cells as well as myoepithelial cells and significant ADPase activity has been reported for microsomes from other tissues. In contrast there are no reports of ADPase in the Golgi apparatus from other tissues, although one other study (23) has suggested that some ADPase activity partially copurified with galactosyl transferase from cultured aortal cells. Note that TPPase activity, a marker of the trans-Golgi, copurifies with galactosyl transferase (Table II). Morphological examination of the Golgi apparatus fraction by electron microscopy showed it to be 85 to 90% pure and relatively free of mitochondria and smaller vesicles. Subcellular fractions were assayed for ADPase at both pH 6.3 and pH 7.4. The activity toward ADP at 7.4 appears to be higher and more concentrated in the Golgi apparatus than the activity which occurs at 6.3.

Maximum velocities and Michaelis constants for membrane-associated mammary ADPases. Experiments were conducted to compare the kinetic constants K_m and V_{max}, for both the Golgi apparatus and microsomes at pH 6.3 and 7.4. ADP has significant association constants for both Ca^{2+} and Mg^{2+} so experiments were designed to test if the substrate for the enzyme(s) might be the metal–ADP complex. When [metal–ADP] was calculated and then varied, increased velocity occurred and Michaelis–Menten behavior was observed, but some of the curves displayed "apparent" cooperativity and were fit better with integer values ($n > 1$) assigned to the concentrations as described in Eq. [3]. The meanings of these exponentials have been discussed (15) and the simplest interpretation is that they are analogous to Hill coefficients. Because Mg^{2+} is used to stabilize the Golgi apparatus, the nonclassical kinetics could be due to residual Mg^{2+}, one preparation was dialyzed against EDTA as described under Materials and Methods, and no changes in the kinetic patterns were observed. Values obtained for K_m and V_{max} of metal–ADP complexes are given in Table III and represent the average of two experiments, one dialyzed and one not dialyzed.

TABLE I

Effect of Triton X-100[a] on Hydrolysis of UDP and ADP by Mammary Membrane-Associated Enzymes[b]

		Golgi apparatus[c]	Microsomes[c]
Activity pH 6.3	UDP	77.3 ± 7.9%	37.8 ± 2.5%
	UDP + Triton	100%	44.7 ± 1.6%
	ADP	27.4 ± 5.9%	35.2 ± 0.7%
	ADP + Triton	6.3 ± 1.0%	3.8 ± 0.5%
	Ratio U/A	2.82	1.07
	U/A + Triton	15.87	11.76
Activity pH 7.4	UDP	44.0 ± 1.2%	51.3 ± 1.9%
	UDP + Triton	36.8 ± 0.3%	31.5 ± 0.65
	ADP	38.7 ± 0.9%	53.9 ± 1.2%
	ADP + Triton	13.4 ± 1.1%	20.4 ± 0.9%
	Ratio U/A	1.14	0.95
	U/A + Triton	2.75	1.54

[a] [Triton X-100] = 0.1%.
[b] Three preparations, three rats each.
[c] Relative activity of UDP + Triton = 100%.

TABLE II

Subcellular Distribution of Mg^{2+} ADPase and Thiamine Pyrophosphatase Activities in the Lactating Rat Mammary Gland

Subcellular fraction	Specific activity (nmol/min/mg protein) ADP, pH 6.3[a]	ADP, pH 7.4[a]	TPP, pH 7.4[a]	Enzyme marker ratio[b]
Homogenate	34.3 ± 3.6	43.1 ± 3.6	17.1 ± 0.6	1.00
Nuclei	24.7 ± 9.7	49.6 ± 12.5	19.2 ± 3.6	—
Mitochondria	49.9 ± 7.9	70.2 ± 4.8	32.9 ± 2.0	5.93[c]
Golgi	39.8 ± 2.8	69.8 ± 0.3	86.4 ± 3.0	28.4[d]
Microsomes	76.2 ± 13.9	112.5 ± 12.1	30.5 ± 1.3	4.26[e]
Cytosol	5.6 ± 0.9	10.5 ± 1.1	10.9 ± 0.9	

[a] Average values for three preparations, three rats each, ±SD.
[b] Average values for four preparations.
[c] Succinate dehydrogenase; ratio of specific activity in mitochondrial fraction to homogenate.
[d] Lactose synthetase; ratio of specific activity in Golgi apparatus fraction to homogenate.
[e] NADPH:cytochrome c reductase; ratio of specific activity in microsomal fraction to homogenate.

TABLE III

Kinetic Parameters for M^{2+}–ADP Complexes for Golgi Apparatus and Micosomal ADPase

	Golgi apparatus			Microsomes		
	K_m (μM)	V_{max} (nmol/min/mg)	Integer (n)	K_m (μM)	V_{max} (nmol/min/mg)	Integer (n)
pH 6.3						
[Mg^{2+}–ADP][a]	25.9 ± 0.1	45.3 ± 2.3	1	15.1 ± 0.1	69.7 ± 7.0	1
[Ca^{2+}–ADP][b]	18.0 ± 1.7	28.9 ± 2.9	1	10.7 ± 0.8	30.5 ± 2.5	1
	76.8 ± 3.8	38.3 ± 2.5	10			
pH 7.4						
[Mg^{2+}–ADP][a]	7.8 ± 1.3	50.0 ± 5.9	2	3.3 ± 0.3	90.5 ± 3.1	2
[Ca^{2+}–ADP][b]	14.6 ± 0.9	65.0 ± 8.0	1	11.7 ± 1.9	77.0 ± 11.0	2

[a] $[Mg^{2+}]_{free}$ maintained at 10 mM; concentration of [Mg–ADP] varied (2 to 200 μM).
[b] $[Ca^{2+}]_{free}$ maintained at 10 mM; concentration of [Ca–ADP] varied (2 to 200 μM).

The K_m values obtained for the metal–ADP complex were much lower than those for ADP alone, indicating that metal–ADP may indeed be the substrate. For the microsomal fraction at pH 7.4 some cooperativity in the metal–ADP reaction is observed (Fig. 3) and $n = 2$ gives the best fits with the lowest root mean square and smallest errors of the coefficients. For both microsomal and Golgi apparatus fractions, the K_m values for Mg^{2+}–ADP (but not Ca^{2+}–ADP) are significantly lower at pH 7.4 than those at pH 6.3. In Fig. 1, where the concentrations of Mg^{2+} and ADP are not calculated, bimodality was also observed. Here for Golgi apparatus and microsomal fractions the V_{max} values remain greater at pH 7.4. For Ca^{2+}–ADP distinct bimodality also occurs (V_{max} at pH 7.4 is greater than that at pH 6.3). Even more intriguing for Ca^{2+}–ADP is the second inflection observed above the calculated concentration of 75 μM (Fig. 4) in the Golgi apparatus at pH 6.3. Such an inflection was not observed for the microsomal fraction, where V_{max} for Ca^{2+}–ADP exhibits simple kinetic behavior at both pH's (Table III). Apparently the Golgi apparatus of the lactating rat mammary gland contains an enzyme able to respond with increased activity at elevated Ca^{2+} and ADP concentrations when the pH drops. If indeed Golgi vesicles mirror milk serum in ionic composition (25), then physiologically the $[Ca^{2+}]_{free}$ in milk (1 to 3 mM) could invoke such a response in the secretory Golgi apparatus and the pH might be expected to be 6.90 or approach 6.75.

Experiments were designed to keep [metal–ADP] constant at five times the K_m values obtained in Table III and the variation of velocity with the free metal ions studied. Whether the fractions were dialyzed or not a residual activity toward ADP was observed in the absence of added metal ions. This activity most likely represents nonspecific acid or alkaline phosphatase in the preparation. For Ca^{2+}–ADP or Mg^{2+}–ADP added metal ions re-

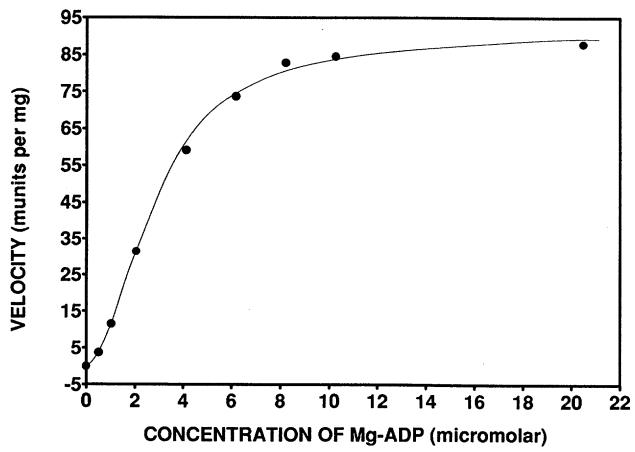

FIG. 3. Velocity (nmol/min/mg) plotted against the calculated concentration of Mg–ADP (μM) for the microsomal fraction. Curve shows "cooperativity" at low concentrations. Data are fitted with Eq. [3]. The assay is as described under Methods and Materials, pH 7.4.

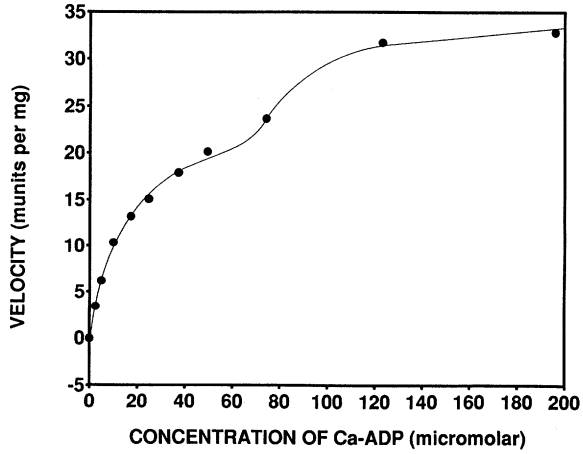

FIG. 4. Velocity (nmol/min/mg) plotted against the calculated concentration of Ca–ADP (μM) for the Golgi fraction. The curve shows two inflection points. Data are fitted with Eq. [3]. The assay is as described under Methods and Materials, pH 6.3.

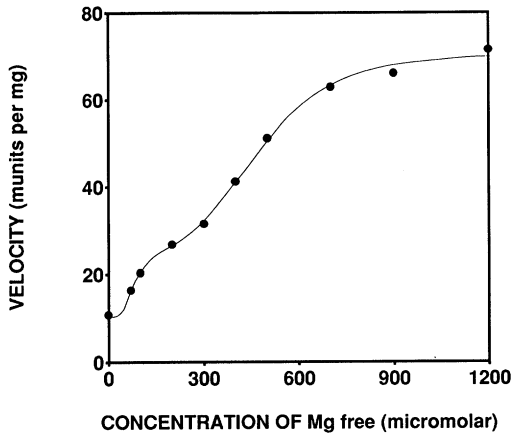

FIG. 5. Velocity (nmol/min/mg) plotted against calculated free Mg^{2+} concentration (μM). The curve shows activity in the absence of added Mg^{2+} and two inflection points. Data are fitted with Eq. [3]. The assay is as described under Materials and Methods using the dialyzed Golgi apparatus, pH 7.4.

sulted in stimulation of activity. At pH 7.4, two distinct inflection points in addition to the baseline values (V_0 of Eqs. [2] and [3]) are observed for the Mg^{2+} experiments in the Golgi apparatus (Fig. 5) and in microsomes. These data were analyzed with Eq. [3] and the results of this analysis are given in Table IV. In both the Golgi apparatus and microsomes at pH 7.4 the values are somewhat similar for the Mg^{2+} and Ca^{2+} experiments, indicating a moderate and a higher K_m site for Mg^{2+} and only one intermediate value for Ca^{2+}. The V_{max} values here are almost all higher at pH 7.4 for the respective experiment carried out at 6.3. At the latter pH, the microsomal kinetic constants mirror those of pH 7.4. For the Golgi apparatus at pH 6.3 K_m values could not be calculated. The smaller increase in activity over the non-metal-stimulated value did not allow a reliable estimate of K_m; however, as shown in Table IV, the metal-stimulated activity remained constant over a wide range of concentrations. These facts could be interpreted in several ways: (a) there is no single measurable binding site at this pH in this system, (b) the change in absorbance is beyond the usable lower range of P_i detectable by the malachite green reagent, (c) metal–ADP hydrolysis is not actually dependent on the free metal at this pH; the last is probably least possible.

The membrane fractions of the lactating rat mammary gland thus contain metal-stimulated ADPases which most likely use metal–ADP complexes as substrates. The concentration range of metal ion stimulation (29 to 787 μM for Mg^{2+} and greater than 100 μM for Ca^{2+}) point to Mg^{2+} as the more likely physiological system, except for the Golgi apparatus, where the $[Ca^{2+}]_{free}$ is thought to be threefold greater than that of $[Mg^{2+}]_{free}$ (25).

Inhibitor studies. Phosphohydrolyases can be classified on the basis of responses to selected inhibitors. Neither the Golgi apparatus nor microsomal fractions were inhibited by ouabain, oligomycin, and levamisole, ruling out Na^+/K^+, F_1/F_0 and F_1 ATPases, and alkaline phosphatase, respectively, as responsible for the ADPase activity observed (Table V). The ADPase activity is typical of a variety of phosphatase enzymes in that both the Golgi apparatus and microsomal fractions are inhibited by vanadate, NaF, and $LaCl_3$. ADPase activity can arise from the action of adenylate kinase (22, 24, 26) but this activity should not liberate inorganic phosphate, the basis for the assay used here. Ap_5A inhibited Golgi and microsomal ADPase 20 and 30%, respectively, arguing against the ADPase activity being due to adenylate kinase. The compound 5′-adenylimidodiphosphate inhibits membrane-

TABLE IV

Kinetic Parameters for $[M^{2+}]_{free}$ for Golgi Apparatus and Microsomal Fractions

	Golgi apparatus			Microsomes		
	K_m (μM)	V_{max} (nmol/min/mg)	Integer (n)	K_m (μM)	V_{max} (nmol/min/mg)	Integer (n)
pH 6.3						
$[Mg^{2+}]$	No Mg^{2+}	16.0 ± 1.2		No Mg^{2+}	6.2 ± 1.9	
	(100 to 900)	35.8 ± 1.1		133 ± 13	48.5 ± 4.1	1
				656 ± 35	60.3 ± 6.2	8
$[Ca^{2+}]$	No Ca^{2+}	19.3 ± 1.2		No Ca^{2+}	6.8 ± 0.5	
	(200 to 800)	59.5 ± 2.0		109 ± 5	51.3 ± 1.9	5
pH 7.4						
$[Mg^{2+}]$	No Mg^{2+}	12.6 ± 1.8		No Mg^{2+}	6.9 ± 1.7	
	74.5 ± 11.0	27.5 ± 1.0	3	29 ± 13	16.9 ± 1.9	1
	497 ± 21.0	74.0 ± 2.9	4	787 ± 23	106 ± 5	3
$[Ca^{2+}]$	No Ca^{2+}	18.5 ± 1.0		No Ca^{2+}	5.5 ± 1.5	
	174 ± 9	97.8 ± 8.1	2	184 ± 8	118 ± 5	2

TABLE V

Effects of Selected Inhibitors on Mg^{2+}-Stimulated ADPase Activity in Golgi Apparatus and Microsomal Fractions[a]

Inhibitor	Concentration	% Activity	
		Golgi apparatus	Microsomal fraction
Control	No additions	100	100
Oubain	5 mM	90	89
Oligomycin	100 μg/ml	72	75
Levamisole	500 μM	94	98
Vanadate	400 μM	53	53
NaF	10 mM	4	3
$LaCl_3$	1 mM	12	7
Ap_5A	0.1 μM	80	70
App[NH]p	200 μM	42	39

[a] Standard assay, pH 7.4; average of three preparations, three rats each.

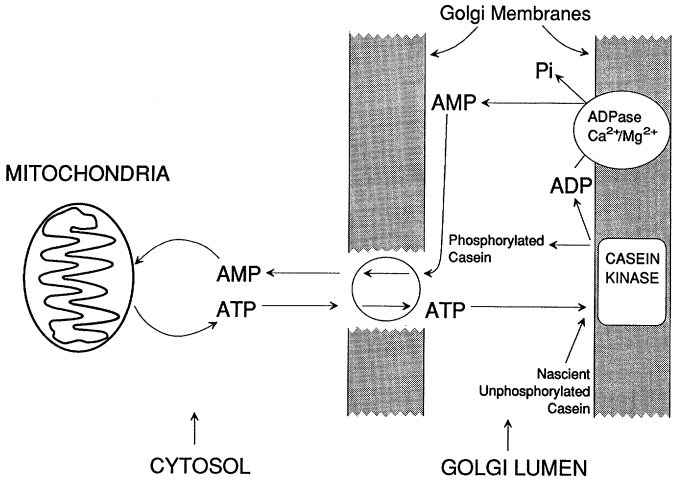

FIG. 6. ATP cycle in the lactating mammary gland. The ADPase described in this manuscript completes the cycle necessary to transport the ATP required to phosphorylate casein in the Golgi apparatus. The ATP ↔ AMP transporter (4) and casein kinase (3) have previously been described.

associated ADPases with $K_i \sim 100$ μM (21, 22, 26). At 200 μM 60% inhibition of ADPase activity was observed for both Golgi apparatus and microsomal fractions of the lactating rat mammary gland. The pattern of inhibitors observed in Table V supports the occurrence of ADPases in the lactating rat mammary gland.

The results presented above suggest strongly the existence of a Ca^{2+}/Mg^{2+} ADPase in the lactating rat mammary gland. Studies on the Golgi apparatus from other tissues (5, 18, 19) have documented the presence of UDP-specific enzymes (which are distinct from ADPase and have been suggested to be a marker for the Golgi apparatus). The UDPase enzyme is stimulated and extracted by Triton X-100. In contrast ADPase activity is inhibited by this detergent (Table I) and exhibits nonlinear kinetics (Fig. 1), perhaps accounting for the fact that it has not been previously reported in the Golgi apparatus.

ADPase activity associated with plasma membranes in a variety of tissues and cells has been reported to occur (Table VI). Its occurrence in mammary gland microsomes is not unexpected considering the various cell types which occur in the gland (myoepithelial and endothelial cells). However, the Golgi apparatus is a distinctive morphological marker for secretory epithelial cells (16). The occurrence of ADPase in the Golgi is a logical extension of one of the more important functions ascribed to this fraction, namely phosphorylation of proteins and in the mammary gland, casein (3). The casein kinase which carries out this function transfers γ-*P* of ATP to specific serine residues (27). For each residue phosphorylated, an ADP is generated. Since Capasso *et al.* (4) demonstrated that the nucleotide transport system in the rat mammary Golgi is selective for AMP and ATP, there is a need for an ADPase enzyme to convert the diphosphate to a monophosphate to complete the ATP cycle depicted in Fig. 6.

Based upon comparison of the properties of the mammary enzyme with the known ADPases it appears (Table VI) as though the Golgi apparatus and microsomal fractions do contain a specific ADPase. The pH profile of the ADPase of the Golgi apparatus is somewhat unique, and such a profile could allow the enzyme to respond to either a basic or the more acidic environment found in milk (28) and presumably secretory ves-

TABLE VI

Comparison of Properties of ADPase Preparations

Source:	Rat lung	Human	Rat heart	Swine aorta	Rat mammary
Subcellular localization	Plasma membrane	Red cell membrane	Sarcolemma	Plasma membrane	Golgi plasma membrane
K_m ADP (μM)	50	28	20	10	10–20
Cations	Mg^{2+}/Ca^{2+}	Mg^{2+}/Ca^{2+}	Mg^{2+}/Ca^{2+}	Mg^{2+}/Ca^{2+}	Mg^{2+}/Ca^{2+}
pH	7.5	7.4	7.4	7.3	6.3/7.4
App[NH]p	60% I	—	60% I	—	60% I
Ap_5A	—	10% I	20% I	—	20/40% I
Levamisole	No I	No I	No I	No I	No I
References	(21)	(24)	(22)	(23)	This study

icles (25). Both Ca^{2+} and Mg^{2+} stimulate activity of enzymes(s) but Ca^{2+} is more likely important in milk secretion, while Mg^{2+} may be related to plasma-membrane-associated interactions. The cooperative interactions of metal ions with enzyme suggest the ability of the ADPase to respond to a wide variety of metal ion fluxes in the Golgi apparatus or in the plasma membranes of the lactating mammary gland.

APPENDIX

Conditions of the Various Assays to Determine the Kinetic Constants of ADPase

	Microsomes		Golgi	
Varied species	Range of varied species[a]	Species held constant	Range of varied species[a]	Species held constant
pH 7.4				
$[Ca]_{free}$	150–1200 μM	[Ca–ADP] = 60 μM	105–1100 μM	[Ca–ADP] = 60 μM
[Ca–ADP]	2.5–196 μM	$[Ca]_{free}$ = 10 mM	2.5–196 μM	$[Ca]_{free}$ = 10 mM
$[Mg]_{free}$	150–1200 μM	[Mg–ADP] = 60 μM	70–1200 μM	[Mg–ADP] = 40 μM
[Mg–ADP]	0.5–21 μM	$[Mg]_{free}$ = 10 mM	2.8–217 μM	$[Mg]_{free}$ = 10 mM
pH 6.3				
$[Ca]_{free}$	100–400 μM	[Ca–ADP] = 50 μM	175–800 μM	[Ca–ADP] = 100 μM
[Ca–ADP]	2.5–196 μM	$[Ca]_{free}$ = 10 mM	2.5–196 μM	$[Ca]_{free}$ = 10 mM
$[Mg]_{free}$	150–1200 μM	[Mg–ADP] = 75 μM	150–900 μM	[Mg–ADP] = 80 μM
[Mg–ADP]	1–80 μM	$[Mg]_{free}$ = 10 mM	2.8–217 μM	$[Mg]_{free}$ = 10 mM

[a] The range of varied species does not include the zero concentration points.

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